PATENT
Docket No.110.01290101

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): O'SULLIVAN
Serial No.: 09/884,894
Confirmation No.:1710
Filed: June 19, 2001
For: BIFIDOBACTERIA AND SIDEROPHORES PRODUCED THEREBY AND METHODS OF USE

Group Art Unit: 1651
Examiner: D.K. Ware

DECLARATION UNDER 37 C.F.R. §1.132

Assistant Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Daniel J. O'Sullivan, declare and say as follows:

1. I am the inventor of the above-identified patent application.

2. I received a Ph.D. in Microbiology from University College, Cork Ireland, in 1990. From 1988-1991 I was a Research Scientist at the National Food Biotechnology Center, Cork, Ireland, and a Research Associate at the Department of Food Science, North Carolina State University, Raleigh, North Carolina, from 1991-1994. I became an Assistant Professor in the Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota, from 1994-2000, and I have been an Associate Professor in the Department of Food Science and Nutrition at the University of Minnesota since 2000. My research activities include analysis of how the expression of commercially significant traits is regulated in the food grade bacteria Lactococcus and Bifidobacterium, and characterizing the complete genome sequence of both these bacteria.

3. I make this Declaration in support of the patentability of the claims of the above-identified patent application.

4. The teachings presented in the above-identified patent application are sufficient to enable a skilled worker to easily and quickly isolate strains of *Bifidobacterium* that produce a siderophore. The Examiner is requested to consider the following experimental data as evidence that the skilled worker, provided with the disclosure in the above-identified application, can practice the invention as broadly claimed.

5. The objective of the following experiment was to obtain an isolate that produced relatively high levels of siderophore for purification purposes. Strains were isolated from 10 subjects essentially as described in Example 1 of the present application. The 10 subjects ranged in age from 2 to 40 and varied in gender, country of origin, diet, and smoking habits. None of the individuals used in Example 1 of the present application were used in the experiment described in this Declaration.

As described in Example 1 of the present application, fresh fecal samples were collected from the subjects, homogenized, transferred to an anaerobic chamber, and serially diluted and plated on BIM-25, a selective media for bifodobacteria. Colonies were selected from the BIM-25 plates, and the colonies were assayed for fructose-6-phosphate phosphoketolase, the diagnostic enzyme for the genus *Bifidobacterium*. Each colony was positive for fructose-6-phosphate phosphoketolase, indicating they were bifidobacteria. Ten isolates, one randomly picked from each subject, were then assayed for the production of a siderophore in a broth system. Selected strains were subsequently speciated using the standard technique of sequence analysis of the 16S rRNA as described in Example 1 of the present application.

Production of the siderophore in broth was accomplished using the procedure described in Example 3. All glassware was soaked in 1 M HCl overnight and rinsed with deionized water prior to use for production of the antimicrobial compound. Each culture was

inoculated from a -70°C stock-culture into 10 ml of TPY broth medium (described at page 20, line 27, through page 21, line 11 of the specification) and incubated anaerobically at 37°C for 48 hours. Freshly grown culture (2%) was re-inoculated into 10 ml of the media described at page 21, lines 15-26 of the specification. After anaerobic incubation for 48 hours at 37°C, the supernatant was collected by centrifugation.

The assay used to detect the production of a siderophore in broth was similar to the assay described at Example 2 of the present specification. Both assay for detecting production of a siderophore in broth and the assay described at Example 2 measure the ability of a *Bifidobacterium* to produce a diffusible compound that inhibits the growth of an indicator strain due to iron competition. The supernatant was filtered through 0.45 µm filter membranes (Millipore corporation, Bedford, MA) and 50 µl of supernatant was tested for its activity using a well diffusion assay. To conduct this assay, a well was cut in a plate containing 1.5% agar and 0.05 mM 2, 2′ dipyridyl, and then 50 µl of supernatant was applied into well. The plate was incubated at room temperature for an hour to allow diffusion of the sample. Plates were overlaid with 4 ml of Brain Heart Infusion (BHI) sloppy agar (0.5% agar) supplemented with 0.35 mM 2, 2′ dipyridyl and 40 µl of freshly grown *Micrococcus luteus*. The plates were examined for the presence and absence of a zone of inhibition against the indicator organism after overnight incubation at 30°C and were compared with the control plate in which 100 µM of iron was added with the indicator organism.

Using this assay, all ten bifidobacteria isolates tested positive for siderophore production. All showed inhibitory zones in the test plates and no zones in the control plates.

6. The assay described at Example 2 of the present application has been used to test 4 of the 10 isolates that are described in paragraph 5 above. As expected, these isolates inhibited the growth of the indicator strain using the assay described at Example 2 of the present application. Moreover, I expect that the siderophore produced by these 10 isolates would also inhibit the growth of the other indicator strains described in the specification.

7. The data disclosed above are proof that the skilled person can easily obtain bifidobacteria strains and determine if they produce a siderophore. These data also show that each time the experimental procedure was conducted with a subject, a bifidobacteria producing a siderophore was obtained. Therefore, using the teachings disclosed in the above-identified application, the skilled worker can practice the invention as broadly claimed.

8. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Tile 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent that issues thereon.

Date: 9-19-03 Signed: Dan O'Sullivan

Daniel J. O'Sullivan